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Characterization of cytosolic phosphoglucoisomerase from immature wheat (*Triticum aestivum* L.) endosperm

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Abstract. Phosphoglucoisomerase from cytosol of immature wheat endosperm was purified 650-fold by ammonium sulphate fractionation, isopropyl alcohol precipitation, DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B. The enzyme, with a molecular weight of about 130,000, exhibited maximum activity at pH 8·1. It showed typical hyperbolic kinetics with both fructose 6-P and glucose 6-P with K_m of 0·18 mM and 0·44mM respectively. On either side of the optimum pH, the enzyme had lower affinity for the substrates. Using glucose 6-P as the substrate, the equilibrium was reached at 27% fructose 6-P and 73% glucose 6-P with an equilibrium constant of 2·7. The ΔF' calculated from the apparent equilibrium constant was +597 cal mol^{-1}. The activation energy calculated from the Arrhenius plot was 5500 cal mol^{-1}. The enzyme was completely inhibited by ribose 5-P, ribulose 5-P and 6-phosphogluconate, with K_i values of 0·17, 0·25 and 0·14 mM respectively. The probable role of the enzyme in starch biosynthesis is discussed.

Keywords. Wheat; *Triticum aestivum;* endosperm; cytosol; phosphoglucoisomerase.

Introduction

Starch is synthesized from sucrose in the endosperm of developing grains (Singh and Mehta, 1986). The process of sucrose-starch conversion has been studied quite extensively in grains of several cereals including wheat, with respect to both changes. in the activities of the enzymes involved and the concentration of metabolites (Kumar and Singh, 1980, 1984). It is believed that the synthesis of starch from sucrose is sequentially indirect, temporally separated and compartmentalized (MacDonald and ap Rees, 1983; Singh and Mehta, 1986). Sucrose, on entering the endosperm, is converted to triose-P in the cytosol, while starch biosynthesis from triose-P occurs in amyloplasts, which are membrane-bound, metabolically active and probably semi-autonomous organelles like chloroplasts (Macherel *et al.,* 1985; Sowokinos *et al.,* 1985; Dowson Day *et al.,* 1988; Entwistle *et al.,* 1988; Ngernprasirtsiri *et al.,* 1988). Hence, in developing wheat endosperm, some of the enzymes of glycolysis and/or gluconeogenesis are present both in cytosol and in amyloplasts (Anand and Singh, 1985; Sangwan and Singh, 1987a, b, 1988). In a recent report, we have shown the presence of two forms of phosphoglucoisomerase (D-glucose-6-phosphate ketoisomerase, EC 5·3·1·9) (PGI) in developing endosperm of wheat (Sangwan and Singh, 1987a). Here we report the detailed kinetic characteristics of the cytosolic form of this enzyme from developing wheat endosperm.

Materials and methods

Chemicals

All the biochemicals used in the present investigation were purchased from Sigma

Abbreviations used: PGI, Phosphoglucoisomerase; TEA, Tris-ethanolamine.

Chemical Co., St Louis, Missouri, USA. All other chemicals used were of high purity analytical grade obtained from BDH and/or E. Merck.

Plant material

Immature wheat grains were harvested from field-grown wheat crop (cv. WH-157) at day 26 after anthesis. Endosperms were removed and stored in liquid nitrogen for further use.

Enzyme purification

Unless stated otherwise, all steps involved in enzyme extraction and purification were carried out at 0–4°C.

Enzyme extraction: Immature wheat endosperms (30g) were homogenized using pestle and mortar in 150 ml of 40 mM Tris–ethanolamine–NaOH (TEA) buffer (pH 7·1). The homogenate was squeezed through 4 layers of cheesecloth and centrifuged at 15,000 *g* for 45 min. The supernatant obtained was employed for ammonium sulphate and isopropyl alcohol fractionations.

$(NH_4)_2SO_4$ *and isopropyl alcohol fractionations*: Solid ammonium sulphate was added to the crude extract to 40% saturation. After 1 h, the precipitate was removed by centrifugation at 15,000 g for 30 min and the supernatant brought to 60% saturation by adding more ammonium sulphate. The precipitate, again removed as above, was dissolved in the minimum amount of extraction buffer and dialysed against 30 mM TEA buffer (pH 7·1) for 48 h. The dialysed ammonium sulphate fraction was then subjected to chilled (–12°C) isopropyl alcohol precipitation between 40 and 65% saturation. The precipitate obtained was collected as above and redissolved in the extraction buffer.

DEAE-cellulose chromatography: The fraction obtained between 40 and 65% saturation of isopropyl alcohol was applied to a DEAE-cellulose column (2·2×30cm) previously equilibrated with the extraction buffer. The column was eluted with about two bed volumes of the buffer, followed by stepwise NaCl gradient elution. Fractions (5 ml each) containing PGI activity were pooled and concentrated by ultra-filtration (Amicon membrane filter).

Sepharose CL-6B chromatography: The above fraction was further purified by passing it through a column of Sepharose CL-6B (2·2×70cm) pre-equilibrated with the extraction buffer. The enzyme was eluted at a flow rate of 20 ml h^{-1} with the equilibration buffer. The active fractions (3 ml each) were pooled and stored at 4°C for further studies.

Assay of PGI

The enzyme was assayed as described earlier (Sangwan and Singh, 1987a). The assay mixture, in a final volume of 1·4 ml, contained: 50 mM Tris-HCl buffer

(pH 8·1), 1 mM fructose 6-P, 0·24 mM $NADP^+$, 2 units of glucose 6-P dehydrogenase and 100 μl of appropriately diluted enzyme preparation. The reaction was started by adding the substrate.

With glucose 6-P, the enzyme was assayed colorimetrically (Takeda *et al.,* 1967). The enzyme activity has been expressed in nkatals. One nkat is defined as the amount of enzyme that transforms one nmol of substrate per second.

Protein was estimated by the method of Lowry *et al.* (1951). However, in chromatography fractions, it was estimated by measuring absorbance at 280 nm.

Results and discussion

The enzyme preparation obtained between 40 and 65% isopropyl alcohol, when chromatographed through a DEAE-cellulose column, was resolved into two peaks of activity. The peak I enzyme did not adsorb to the column, whereas the peak II enzyme adsorbed and was eluted with elution buffer containing 0·1 M NaCl. No further enzyme activity was eluted up to 0·5 M NaCl. On rechromatography, the two peaks eluted at their respective salt concentration. The relative proportions (9:1) of the two forms did not change after incubating the homogenate at room temperature for various periods of time, thus excluding the possibility that the enzyme heterogeneity was due to proteolysis. Based on physical and kinetic characteristics, peak I enzyme was identified as cytosolic and peak II enzyme as amyloplastic PGI (Sangwan and Singh, 1987a). The cytosolic form, when further passed through a column of Sepharose CL-6B, yielded specific activity that was 650-fold higher than that of the crude homogenate (table 1). These results have been described in detail in our earlier report (Sangwan and Singh, 1987a).

Table 1. Purification of cytosolic PIG from immature wheat endosperm.

Fraction	Total activity (nkats)	Total protein (mg)	Sp. activity (nkats/mg protein)	Fold purification	Yield (%)
Crude extract	8688	1689	5·1	—	100
Ammonium sulphate	7819	284·2	27·5	5·4	90·0
Isopropyl alcohol	6519	99·2	65·7	12·9	75·0
DEAE-cellulose	3848	1·99	1938·8	380	44·3
Sepharose CL-6B	3531	1·063	3321·1	651	40·6

The enzyme was stable for more than a month at 4°C. It did not lose any activity on incubation for 15 min at 40°C. However, at higher temperatures, the enzyme was not stable and lost.50% of the activity at 45°C. At 55°C, the enzyme was completely inactivated. The activation energy, calculated from the Arrhenius plot, was 5500 cal mol^{-1}.

The enzyme had a molecular weight of about 130,000, as determined by gel filtration chromatography. A similar value (120,000–130,000) has been reported for the enzyme from germinating and developing *Cassia* seeds (Lee and Matheson, 1984), castorbean endosperm (Dennis *et al.,* 1985) and spinach leaves (Schnarrenberger and Oeser, 1974).

The enzyme was optimally active at pH 8·1 (figure 1). It had much lower activity

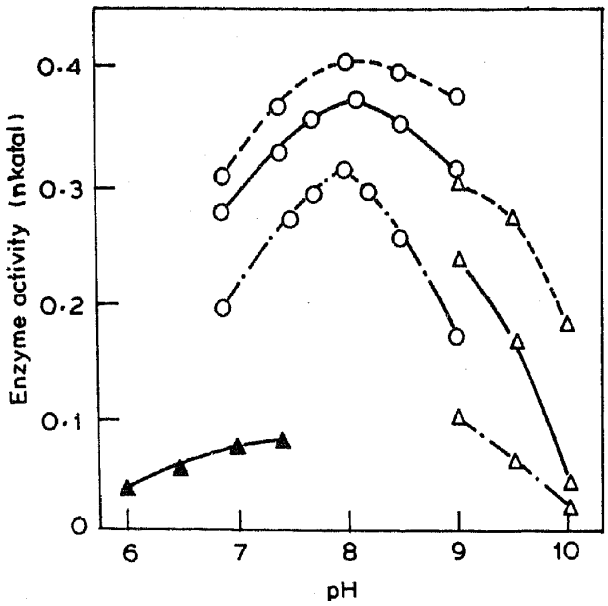


Figure 1. pH optimum curves of PG1 at different substrate (fructose 6-P) concentrations: 0·4 mM (**.....**), 1·0 mM (——) and 3·0 mM (– – – –). Buffers: Tris–maleate (▲), Tris–HC1 (O), glycine–NaOH (Δ).

in Tris–maleate buffer, as has been shown earlier for pea seed enzyme (Takeda *et al.*, 1967). The enzyme from developing Cassia seeds had good activity over a broad pH range (6–11). However, the spinach leaf enzyme had different pH optima with different substrates—7·6 with fructose 6-P and 8·6 with glucose 6-P (Schnarrenberger and Oeser, 1974).

At its optimal pH, the enzyme showed normal Michaelis–Menten kinetics with either fructose 6-P or glucose 6-P as the substrate. However, the enzyme had higher affinity for fructose 6-P than for glucose 6-P. The K_m values determined from Lineweaver–Burk plots were 0·18 and 0·44 mM respectively for fructose 6-P and glucose 6-P. These values are comparable with those reported for the enzyme from germinating Cassia seeds (Lee and Matheson, 1984) and sweet potato (Sasaki *et al.*, 1972). However, much higher values have also been reported for the enzyme from spinach leaves (Schnarrenberger and Oeser, 1974). There was a great influence of pH on K_m for fructose 6-P. Suboptimal pH, on either side of the optimum, increased. K_m and decreased V_{max} (figure 2). The effect was more pronounced at alkaline pH than at acidic pH. The equilibrium constant calculated from K_m and V_{max} in both directions approached the ratio of 3:1, which is similar to the one reported for the enzyme from animal sources (Kahana et al., 1960).

Figure 3 shows the equilibrium at 30°C using glucose 6-P as the substrate. The equilibrium was reached at 27% fructose 6-P and 73% glucose 6-P. The pea seed enzyme showed equilibrium at 35% fructose 6-P and 65% glucose 6-P (Takeda *et al.*, 1967). The apparent free energy change (ΔF') for formation of fructose 6-P, calculated from the apparent equilibrium constant (K_{eq} = F6P/G6P), was found to be +597 cal $moil^{-1}$. The value is rather high compared to that reported for pea seed enzyme (Takeda *et al.*, 1967).

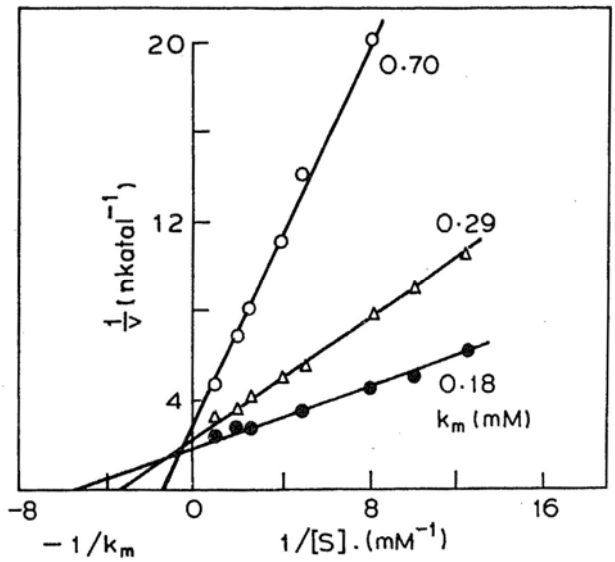


Figure 2. Effect of pH on K_m of PGI for fructose 6-p. Tris-HCl, pH 7 (Δ), Tris–HCl, pH 8·1 (●), and glycine-NaOH, pH 9·5 (O).

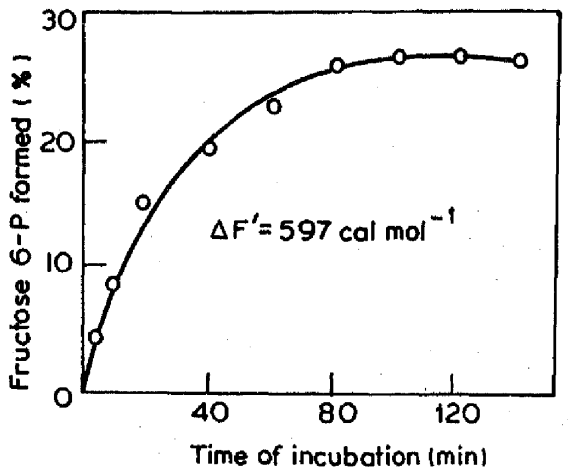


Figure 3. Equilibrium of interconversion of fructose 6-P and glucose 6-P by PGI at pH 8·1 and 30°C. The enzyme was incubated with 5 mM glucose 6-P.

The enzyme was inhibited competitively by 6-phosphogluconate (figured 4), ribose 5-P and ribulose 5-P, with K_i values, determined by Dixon's plot, of 0·14, 0·25 and 0·17 mM respectively. The enzymes from maize endosperm (Salamini *et al.,* 1972), *Cassia* (Lee and Matheson, 1984) and pea seeds (Takeda *et al.,* 1967) are also known to be inhibited by 6-phosphogluconate.

The organic acids succinate, acetate, citrate, tartarate, lactate, fumarate, glutamate, aspartate, glycollate, *β*-hydroxybutyrate, pyruvate and malate had no effect on the activity of the enzyme up to 10 mM. However, at this concentration, 2-oxoglutarate, oxaloacetate, malonate and maleate inhibited the activity by 20, 25, 15 and 50% respectively. ATP, ADP, AMP, phospho (enol) pyruvate and 3-phos-

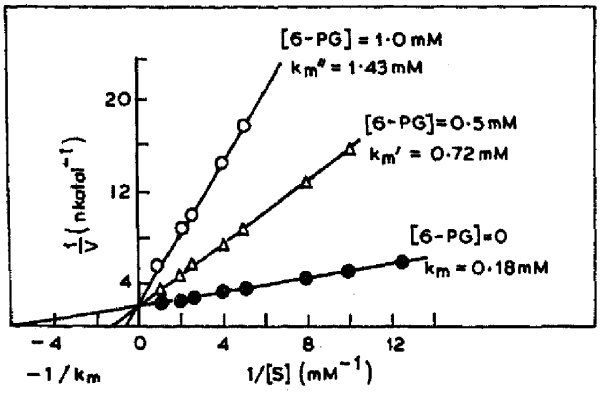


Figure 4. Competitive inhibition of purified PGI at various fixed concentrations of 6-phosphogluconate, with varying fructose 6-P concentrations.

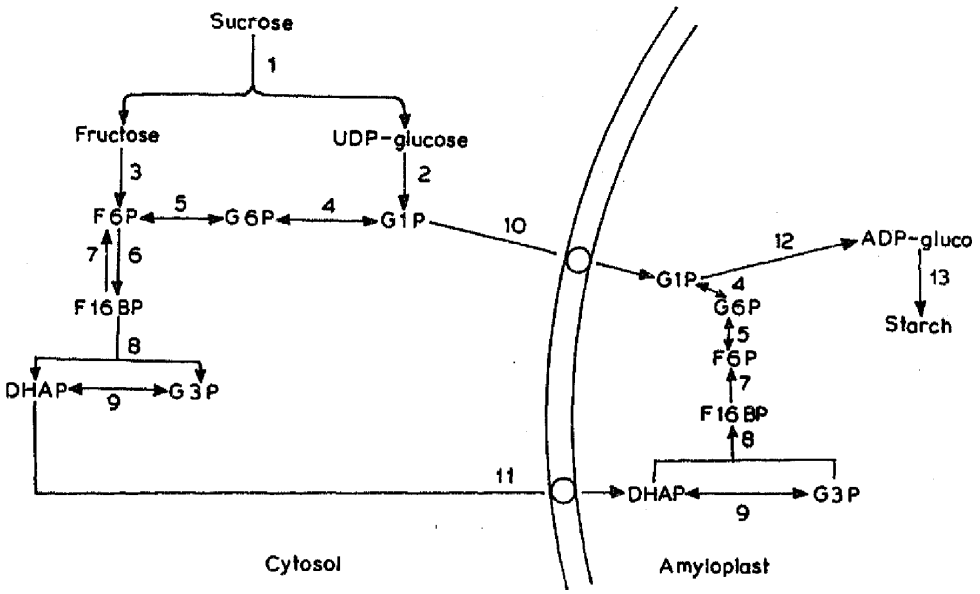
phoglyceric acid at 1 mM concentration had no effect on the activity of the enzyme. Similarly, glucose 1, 6-bisphosphate and fructose 2,6-bisphosphate at 50 μm concentration did not inhibit the enzyme.

The monovalent cations K^+, Na^+ and NH_4^+ (at 10 mM) did not influence the activity of the enzyme. The activity of the lucerne enzyme was also not affected by monovalent cations (McCleary and Matheson, 1976). Among the divalent cations, Mg^{2+}, Ca^{2+} and Ba^{2+} had no effect, whereas Cd^{2+}, Mn^{2+} and Zn^{2+} inhibited the enzyme to the extent of 15–20%, Sr^{2+} and Ni^{2+} inhibited the activity to the extent of about 45%. Hg^{2+} at 10 mM caused 80% inhibition. However, the lucerne enzyme was shown to be completely inactivated by 1 mM of Hg^{2+}. The maize enzyme has also been shown to be inhibited by Zn^{2+} (Salamini *et al.*, 1972).

The enzyme was insensitive to 10 mM concentration of Cl^-, Br^-, CO_3^- and SO_4^{2-}. However, inorganic phosphate and pyrophosphate could inhibit the enzyme to the extent of about 35%.

According to the currently held view, starch in the endosperm of developing grains is synthesized in the amyloplasts (Singh and Mehta, 1986). Sucrose, on entering the endosperm, is converted to fructose and UDP-glucose by sucrose synthase. Both these products are ultimately converted to fructose 6-P, the former by the action of fructokinase and the latter by the combined action of UDP-glucose pyrophosphorylase, phosphoglucomutase and phosphohexose isomerase. Fructose 6-P is then converted to triose-P by the well-known reactions of glycolysis in the cytosol. Triose-P then enters the amyloplasts *via* the phosphate translocator and is then ultimately converted to glucose 1-P by the reverse reactions of glycolysis. Glucose 1-P in plastids is converted to the precursor of starch synthesis (ADP-glucose) by the action of ADP-glucose pyrophosphorylase. All these enzymes have now been shown to be present in developing endosperm of cereal grains (Singh and Mehta, 1986) including wheat (Kumar and Singh, 1980, 1984) (figure 5).

According to the above scheme of reactions, we must expect at least two isoenzymic forms of triose phosphate isomerase, aldolase, fructose-1,6-bisphosphatase, phosphohexose isomerase and phosphoglucomutase. Such distribution of isoenzymic forms has now been well documented for the developing endosperm of wheat (Anand and Singh, 1985; Sangwan and Singh, 1987a, b; Sangwan and Singh,



Figured 5. Proposed metabolic pathway of starch synthesis in amyloplasts of developing cereal grains. 1, Sucrose synthase; 2, UDP-glucose pyrophosphorylase; 3, hexokinase; 4, phosphoglucomutase; 5, phosphohexose isomerase; 6, ATP-dependent phosphofructokinase; 7, fructose-1, 6-bisphosphatase; 8, aldolase; 9, triose-phosphate isomerase; 10, hexose translocator; 11, phosphate translocator; 12, ADP-glucose pyrophosphorylase; 13, starch synthase.

1988). The cytosolic form of PGI may thus be involved in converting the glucose moiety of the incoming sucrose to fructose 6-P according to the scheme given above. Higher affinity of the enzyme for fructose 6-P will favour the latter's conversion to glucose 6-P, which could ultimately serve as the source of carbon for synthesis of starch in amyloplasts through conversion to glucose 1-P, which enters the amyloplast possibly through a hexose translocator (Keeling *et al.,* 1988; Tyson and ap Rees, 1988). Inhibition of the enzyme by 6-phosphogluconate, ribose 5-P and ribulose 5-P suggests a close relationship between the above set of reactions and the oxidative pentose phosphate pathway. Accumulation of intermediates of the oxidative pentose phosphate pathway will block the above set of reactions at the level of PGI. Once this route is blocked, the intermediates of the oxidative pentose phosphate pathway will be recycled for the production of fructose 6-P through the combined actions of ribose phosphate isomerase, ribulose phosphate 3-epimerase, transketolase and transaldolase, etc. Hence, after producing reducing power, the intermediates of this pathway may be utilized for providing energy and extra carbon for onward transport to plastids for the synthesis of starch. Thus, the pathway could play a role in regulating the proportion of carbon shuttled through glycolytic and gluconeogenic reactions.

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